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1. Defective intracellular activity of GDP-D-mannose-4,6-dehydratase in leukocyte adhesion deficiency type II syndrome.
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CS Zentrum Biochemie und Molekulare Zellbiologie, Georg-August-Universitat, Gottingen, Germany.

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Defective intracellular activity of GDP-D-mannose-4,6-dehydratase in leukocyte adhesion deficiency type II syndrome

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Abstract **Leukocyte adhesion deficiency type II (LAD II) is a rare genetic disease characterized by severe immunodeficiency which is related to defective expression in leukocytes of sialyl-Lewis X (SLeX), a fucosylated ligand for endothelial selectins. The molecular basis of LAD II is still unknown, but has been tentatively localized in the de novo pathway of GDP-L-fucose biosynthesis from GDP-D-mannose. Here, we demonstrate that in cell lysates from a LAD II patient, GDP-D-mannose-4,6-dehydratase (GMD), the first of the two enzymes of the pathway has a defective activity compared to control subjects. GMD in cell lysates from both parents showed intermediate activity levels. Cloning of GMD from patient and control lymphocytes ruled out any mutation affecting the amino acid GMD sequence and the purified recombinant proteins from both controls and the patient showed identical specific activities. Since the levels of immunoreactive GMD in cell lysates were comparable in the patient and in controls, the biochemical deficiency of intracellular GMD activity in LAD II seems to be due to mutation(s) affecting some still unidentified GMD-regulating protein.**

Key words: L-Fucose; GDP-D-mannose-4,6-dehydratase; Leukocyte adhesion deficiency type II; Sialyl-Lewis X; Lymphocyte

1. Introduction

Leukocyte adhesion deficiency type II (LAD II) is an autosomal recessive congenital disease, characterized by immunodeficiency and severe mental and growth retardation [1,2]. These patients also display a Bombay phenotype and lack of sialyl-Lewis X (SLeX) antigen on the leukocyte surface [1]. SLeX is a fucose-containing glycoconjugate ligand for the selectins expressed on endothelial cells and on lymphocytes [3–7]. The interaction between SLeX and selectins is the first of a series of events mediating leukocyte rolling and the subsequent extravasation to start the inflammatory response [7]. Granulocytes from LAD II patients have been demonstrated to fail both to interact with cytokine-activated endothelial cells and to bind to recombinant E-selectin in vitro [8,9]. Moreover, in vivo experiments in the patients revealed impaired chemotaxis, reduced half-life and increased turnover rate of neutrophils [10]. These defects in granulocyte functions have been related to the lack of SLeX expression on leukocytes, which prevents interaction with selectins, thus inhibiting their tissue extravasation and causing the immunodeficiency.

The widespread lack of L-fucose on several different glycoconjugates seems to exclude any impairment of fucosyltransferase activities. Rather, it has been proposed that the adhesion deficiency is due to a general defect in L-fucose metabolism [1,2], in particular in the intracellular production of GDP-L-fucose, the substrate of fucosyltransferase activities. However, the molecular basis of the LAD II syndrome is still unknown. In normal cells, GDP-L-fucose is produced either by a de novo pathway starting from GDP-D-mannose or from a quantitatively minor pathway of L-fucose salvage (for a review see [11]). Cytosolic GDP-L-fucose is then transported across Golgi membranes by a specific translocation system [12] and donates the L-fucose moiety to several glycoconjugate acceptors via different fucosyltransferases [6,13]. The major de novo biosynthetic pathway, initially described by Ginsburg in the 1960s [14,15], involves first a dehydration of GDP-D-mannose to GDP-4-keto-6-deoxy-D-mannose, catalyzed by a GDP-D-mannose 4,6 dehydratase (GMD), and then a double epimerase and NADPH-dependent reductase reaction, catalyzed by a single protein. GMD has been identified and characterized in several organisms, from *Escherichia coli* [16] to human cells [17], and it proves to be very well conserved during evolution, with more than 60% amino acid identity between the bacterial and human enzymes. The double epimerase/reductase activity of the second enzyme has been initially proposed by Chang et al. [18] for the protein purified form porcine thyroid. It was then confirmed with the protein purified in our laboratory from human erythrocytes [19], which has been identified with the human FX protein [20,21], and also with the recombinant *E. coli* protein [22]. Cloning of both human enzyme proteins involved in the de novo pathway, i.e. GMD and FX, allowed us to address the site of LAD II deficiency at the biochemical level. During the present study, human GMD was cloned from a HL-60 promyelocytic cell line and its derived amino acid sequence turned out to be identical to that independently obtained in our laboratory [17]. The results reported in this paper unequivocally identify the reaction catalyzed by GMD as the affected step in LAD II cells. Interestingly, no mutation was found in the coding region of patient GMD cDNA and no quantitative alterations of the protein seem to be present. These data suggest that the molecular basis of this genetic disorder, resulting in a defective biosynthesis of cytosolic

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Abbreviations: GMD, GDP-D-mannose-4,6-dehydratase; LAD II, leukocyte adhesion deficiency type II; SLeX, sialyl-Lewis X; PHA-M, phytohemagglutinin M; EBV, Epstein-Barr virus; FCS, fetal calf serum; PMSF, phenylmethylsulfonyl fluoride; AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; ECL, enhanced chemiluminescence; IPTG, isopropyl-β-thiogalactopyranoside

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GDP-L-fucose, is at the level of some still unidentified factors regulating intracellular GMD activity.

2. Materials and methods

2.1. Cell isolation and culture

Peripheral lymphocytes from the LAD II patient, from his parents and from healthy subjects were purified from total heparinized venous blood using the Ficoll-Hypaque procedure (Pharmacia Biotech, Uppsala, Sweden). Informed consent for the use of samples was obtained from the patient's parents and from all other subjects involved in this study. After purification, lymphocytes were stimulated using 0.5% PHA-M (Sigma Chemicals, St. Louis, MO, USA) and 100 U/ml human recombinant IL-2 (Genzyme, Cambridge, MA, USA) in RPMI 1640 medium containing 10% FCS and 5 mM glutamine (HyClone, Logan, UT, USA). After 3 days of stimulation, PHA-M was removed and cells were cultured in RPMI medium supplemented with 100 U/ml IL-2. Alternatively, B-lymphocytes were transformed with Epstein-Barr virus (EBV) following published protocols [23]. Transformed cells were then maintained in RPMI 1640 medium. All cultures were routinely screened for mycoplasma contamination.

2.2. Preparation of cell lysates and determination of enzymatic activities

IL-2 stimulated lymphoblasts and EBV-transformed cells were harvested during exponential growth and washed twice with ice-cold PBS. Cell pellets were resuspended at 100×10^6/ml in 100 mM sodium phosphate buffer, pH 8.0, containing 0.1 mM PMSF, 40 μM AEBSF, 0.1 mM leupeptin and 50 μM pepstatin. Cells were lysed in ice using a Dounce homogenizer with 30 strokes and were then centrifuged at $10000\times g$ for 20 min a 4°C. The supernatants were centrifuged again at $100000\times g$ for 60 min at 4°C. All fractions obtained were used to assay GMD activity. Protein concentration was determined according to Bradford [24].

The activity of both individual enzymes involved in the conversion of GDP-D-mannose to GDP-L-fucose, i.e. GMD and FX, and the overall activity of the complete pathway were assayed as previously described [16,19]. The activity of GMD on the cytosolic fraction was determined in the absence of NADPH, thus preventing FX activity and the production of GDP-L-fucose. Conversely, FX activity was analyzed using as substrate the intermediate compound GDP-4-keto-6-deoxy-D-mannose, which was produced using the bacterial recombinant GMD [16].

SDS-PAGE and Western blot analyses on the subcellular fractions were performed as described [25,26], using a rabbit polyclonal anti-bacterial GMD antibody, which was observed to recognize also the human enzyme. Detection was performed with a second peroxidase-labeled anti-rabbit IgG antibody using the ECL system (Amersham Life Sciences, Milan, Italy), following the supplier's instructions.

2.3. Purification and sequencing of mRNA

Patient and control RNAs were purified from both normal and EBV-transformed lymphocytes using Trizol reagent (Life Technologies Italia, Milan, Italy). Total RNA was retrotranscribed using MuMV-RT (Promega, Madison, WI, USA) as described [27], using either oligo dT or gene-specific primers designed on human GMD sequence (GenBank accession number AF042377), which were obtained by TibMolBiol (Genoa, Italy). PCR amplification of cDNA with GMD gene-specific primers was performed in the following conditions: 1×Thermo buffer (Promega), 1.5 mM $MgCl_2$, 200 μM dNTP (Pharmacia), 40 μM primers and 2.5 U Taq polymerase (Promega). The results of amplification were analyzed by agarose gel electrophoresis and ethidium bromide staining. Bands corresponding to the expected size were cut from the agarose gel and the amplification products were then purified using the Nucleospin Extract kit (Macherey-Nagel, Düren, Germany). After purification, PCR products were ligated in pMOS*Blue* vectors, using the pMOS*Blue* Blunt End cloning kit (Amersham). Plasmids containing the correct inserts were purified using High Pure Plasmid Isolation (Boehringer Mannheim, Milan, Italy) and sequenced, either manually with Sequenase 2.0 (Amersham) or using the Applied Biosystem 373A automatic sequencer.

2.4. Expression of the recombinant proteins

GMD cloned from normal and LAD II patient were expressed in *E. coli* as fusion proteins with GST, using pGEX-6P1 vector (Pharmacia). The coding region of GMD was amplified using two specific primers containing *Bam*HI (forward) and *Eco*RI (reverse) restriction sites. The first methionine, corresponding to nucleotide 76 of the published sequence [17], was considered the starting residue. The amplified product was cut with the appropriate restriction enzymes, ligated in pGEX-6P1 vector and the ligation product was used to transform competent K803 cells. DNA sequence was performed on plasmids purified from clones positive to PCR screening. Expression and affinity purification with GSH-Sepharose (Pharmacia) of the recombinant proteins were performed as described [16], with slight modifications. Briefly, bacterial culture was induced at an optical density of 0.6 at 660 nm, using 0.2 mM IPTG and incubated for 4 h at 25°C. Cleavage of the native GMD from the matrix(GSH-Sepharose)-bound fusion protein was achieved using Prescission Protease (Pharmacia), upon incubating at 4°C for 16 h. Enzymatic activities of the control and patient GMD were assayed as previously reported for the bacterial recombinant GMD [16].

Recombinant human FX protein was produced as fusion protein with GST, using pGEX-4T2 vector (Pharmacia). The coding region of placental FX cDNA (GenBank accession number U58766) was amplified by PCR, using gene-specific primers containing *Bgl*II (forward) and *Eco*RI (reverse) cleavage sites. The digested product was ligated to *Bam*HII and *Eco*RI cleaved sites of pGEX-4T2 and used for transformation of competent K803 cells. Conditions of expression and purification were as described [16]. The native protein was obtained after incubation with thrombin protease (Pharmacia) for 16 h at 25°C.

3. Results

GMD activity was first comparatively assayed in lysates of PHA plus IL-2-stimulated lymphoblasts from the LAD II patient and from a normal healthy subject. As shown in Fig. 1, GMD activity was remarkably lower in the LAD II patient than in the control subject, who was recruited from the same geographical area of the patient and whose GMD activity was comparable to that observed in other healthy subjects examined in our laboratory (not shown). Another consistent difference between the two samples was the time-dependent trend of patient GMD to regain activity as compared to the control which showed conversely linear patterns of enzymatic activity during incubation. This peculiar deviation from linearity was also observed in lysates of EBV-transformed lymphocytes from the LAD II patient (Fig. 2A). In these immortalized cells, deficiency of GMD activity as compared to the control was less pronounced than in the IL-2

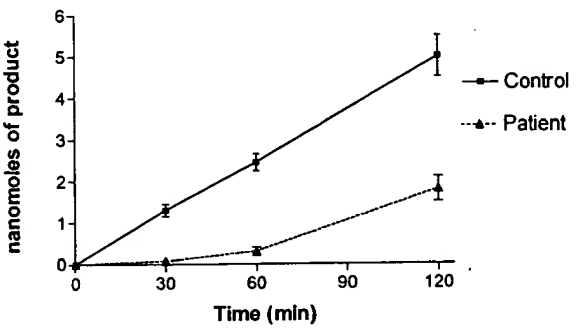

Fig. 1. GDP-D-mannose conversion to the product GDP-4-keto-6-deoxy-D-mannose by GMD in control and LAD II patient cytosolic fractions of PHA+IL-2-stimulated lymphoblasts. Incubations were performed for the times indicated at 37°C with 50 μM GDP-D-[^{14}C-U]mannose, using 1 mg/ml of cytosolic protein. Conditions of incubation and determination of the product GDP-4-keto-6-deoxy-D-mannose by HPLC analysis were as described [16]. Results shown are mean ± S.D. of three separate experiments.

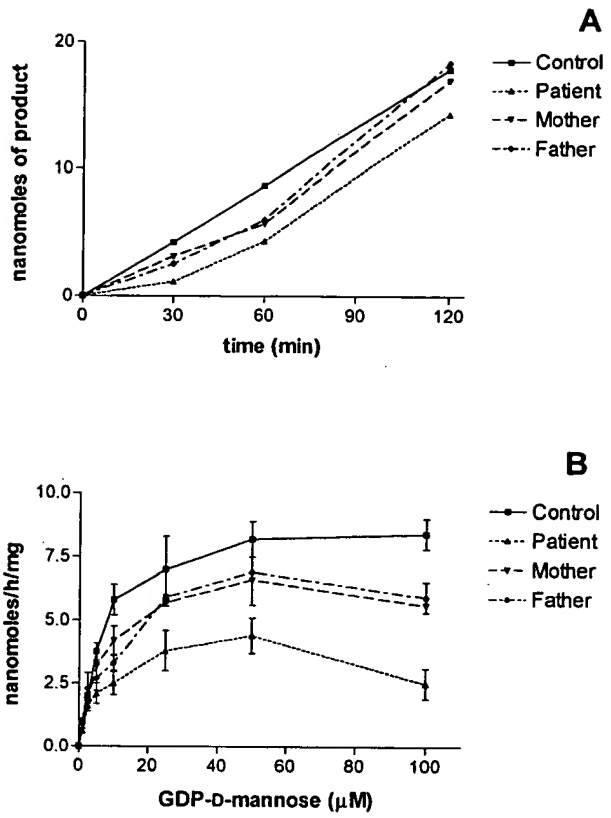

Fig. 2. A: Activity of GMD in the cytosolic fraction of EBV-transformed lymphocytes during incubation at 37°C with 50 μM GDP-D-[^{14}C-U]mannose, using 1 mg/ml of protein. Data from a representative experiment are shown. B: Specific activities of GMD in the cytosolic fraction of EBV-transformed lymphocytes from control, the LAD II patient and his parents as function of GDP-D-mannose concentrations. Results are mean ± S.D. of four separate experiments.

treated lymphoblasts (Fig. 1). However, a significant increase in GMD activity (about four-fold) was also observed in control EBV-transformed cells over that of the IL-2 stimulated lymphoblasts. EBV-transformed lymphocytes from both parents of the patient featured GMD activity levels intermediate between those of the patient himself and the control cells. The corresponding time-dependent profiles of activity were closer to the linear patterns observed in the control immortalized cells than to the upward concave behavior of the patient cells, especially at the lower concentrations of substrate (not shown).

Fig. 2B illustrates the substrate saturation curves of GMD activity in lysates of immortalized lymphocytes from the LAD II patient, from his parents and from a representative control subject. While a clear deficiency of GMD activity was observed in the LAD II patient cells as compared to the control transformed lymphocytes, the cells from both father and mother displayed intermediate activity levels. Apparently, no differences were observed in the K_m values of GMD from the four different samples, which were around 5 μM GDP-D-mannose in all cases. However, another distinctive property of the patient GMD was a remarkable inhibition by high substrate concentrations, which could never be observed in control cells, while its extent was comparatively and equally lower in EBV-transformed lymphocytes from both father and mother (Fig. 2B). FX levels of enzymatic activity were comparable in all

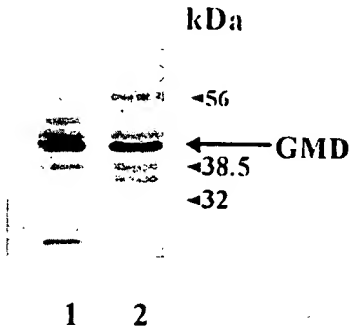

Fig. 3. Western blot analysis on the cytosolic fraction of patient (lane 1) and control (lane 2) lymphoblasts using a polyclonal anti-GMD antibody. 50 μg of total protein was loaded onto each lane and subjected to SDS-PAGE under reducing conditions on a 12% acrylamide gel [25] and to blotting on nitrocellulose membrane. Detection was achieved by the ECL method.

examined samples (84 ± 5.6 nmol/h/mg cytosolic protein), thus excluding any parallel involvement also of this enzyme in LAD II.

GMDs from both patient and control were then investigated by Western blot analyses of the corresponding cytosolic fractions, using a rabbit polyclonal antibody raised against recombinant *E. coli* GMD, which, due to the presence of a high sequence homology [17], is able to cross-react also with the human protein. As shown in Fig. 3, a band corresponding to GMD was also present in the patient cells, in amounts comparable to those found in the control. An additional immunoreactive band of approximately 20 kDa was also observed in patient lymphocytes, but it is still unclear whether this represents a shorter form of the protein or a possible product of degradation.

Sequence of the coding region of the GMD cDNA obtained from the patient cells consistently revealed that no amino acid substitution is present in the patient, compared to the published sequence obtained from HL-60 cell line [17] and to the sequence concurrently and independently achieved in our lab-

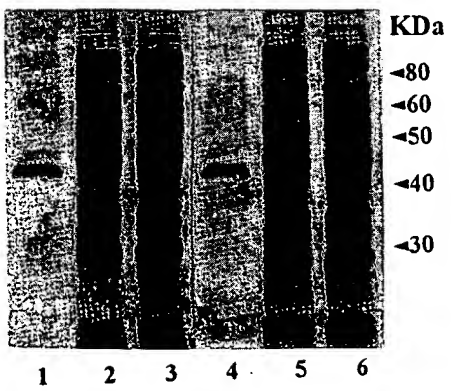

Fig. 4. SDS-PAGE analysis [25] of recombinant GMD from patient (lanes 1, 2 and 3) and control (lanes 4, 5 and 6) lymphocytes, expressed as fusion proteins with GST. Lanes 1 and 4, purified GMD recovered after Prescission protease cleavage; lanes 2 and 5, effluent from GST-Sepharose; lanes 3 and 6, bacterial cytosolic fraction recovered after cell lysis and centrifugation at 14000×*g*. The gel was stained with Coomassie brilliant blue. After cleavage GMD migrated with an apparent M_r of approximately 43 kDa compared to known standards, consistent with the expected size derived from the amino acid sequence.

oratory upon cloning GMD from normal human lymphocytes.

Another unequivocal indication that the defect in GMD activity observed in cell lysates from patient cells is not due to a qualitatively modified protein came from the expression and comparative assay of specific enzymatic activities of the normal enzyme and the patient GMD, both derived form lymphocytes as recombinant proteins (see Section 2.4). These were obtained in a pure form (Fig. 4, lanes 1 and 4) and displayed a comparable GMD activity of 42 ± 3 μmol/h/mg of protein. The K_m values were 3.0 ± 0.5 μM GDP-D-mannose for both patient and control recombinant GMDs. Addition of cofactors, such as NAD^+ or $NADP^+$, had no effect whatsoever on the activity of both enzymes. Recombinant human FX was added at the end of incubation with both control and patient GMDs and led to the complete disappearance of the intermediate compound formed, thus indicating that the product of two enzyme proteins is indeed GDP-4-keto-6-deoxy-D-mannose.

4. Discussion

GMD from cells of the LAD II patient under study showed rather unconventional kinetic properties as compared with the enzyme from control cells. Deviation of enzymatic activity from linearity during incubation would suggest some mechanisms of time-dependent activation which remain so far unidentified and which do not hold at all for the wild type GMD. Likewise, the peculiarity of GMD inhibition by high substrate concentrations, which was observed in the patient cells only, seems to indicate a similarly altered catalytic mechanism. Both kinetic anomalies of GMD, i.e. apparent self-activation and inhibition by high substrate, were maintained, although to a comparatively lower extent, in the cells of the two heterozygous LAD II subjects that were investigated in parallel. GMD protein, as determined by Western blot experiments, was apparently present in patient cells in amounts not lower than in the control cells, thus ruling out the hypothesis of a defective activity due to reduced intracellular levels of the enzyme protein.

Even though the data obtained strongly suggest the impairment of GMD activity in the molecular pathogenesis of LAD II syndrome, sequence analysis of cDNA and kinetic parameters of the recombinant GMD in the patient seem to exclude the presence of a mutation either affecting the catalytic activity of the enzyme or responsible for a lower enzyme stability. Mutations in the non-coding regions of the gene, potentially affecting mRNA expression, stability or translation, can be similarly excluded by the finding of comparable amounts of GMD protein in the cytosolic fraction from both control and patient cells. Moreover, the unusual kinetic properties shown by GMD in the cytosolic fraction of patient cells cannot be obviously explained by reduced levels of the protein.

The only likely explanation for an enzyme deficiency associated with normal intracellular levels of a structurally normal enzyme protein seems to be a genetic mutation in an as yet unknown protein involved in the control of GMD activity, which can be altered following cell disruption and the subsequent enzymatic assay in vitro, thus explaining the apparent self-activation consistently observed during incubation.

Further studies are required to identify possible regulatory mechanisms of GMD, both in normal and pathological conditions, such as LAD II syndrome or other diseases in which an alteration in L-fucose metabolism seems to be involved, e.g. advanced neoplastic or inflammatory diseases [28–30]. Of particular interest is the observation that after immortalization of B-lymphocytes with EBV, all cell lines are characterized by a significant increase in GMD activity, compared to that observed after PHA+IL-2 stimulation. It is still unclear whether these quantitative differences could be due to the presence of different lymphocyte populations or, alternatively, be an effect related to cell immortalization. Since GDP-L-fucose is the substrate of fucosyltransferases, elucidation of regulatory mechanisms of the metabolic pathway leading to its production, and especially of its rate-limiting reaction, could give further insights also into the complex regulation of biosynthesis and remodeling of fucosylated glycoconjugates.

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